

Immunocytochemical Evidence for the Involvement of RFamide-like Peptide in the Neural Control of Cricket Accessory Gland

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ABSTRACT—Using anti-RFamide antiserum, innervation of the accessory gland of the male cricket was investigated. RFamide-like immunoreactive fibers were found over the musculature of this organ, i.e., in the thin muscle winding around each glandular tubule as well as in the thick muscle surrounding the opening of each tubule to the ejaculatory duct. This result suggests that in addition to the previously reported proctolinergic system, another peptidergic system mediated by an RFamide-like substance is involved in regulation or modulation of contractile activity of the accessory gland.

INTRODUCTION

Spermatophore formation in the male cricket requires the rapid release of ingredients produced by the accessory gland at a certain period in the mating cycle [7]. This implies that contraction of the muscles of the accessory gland to extrude the secretions may be directly triggered by neural signals. In our previous morphological and electrophysiological investigations it was shown that the accessory gland is innervated by two types of neurons originating in the terminal abdominal ganglion [5, 11, 12]. The first type is the dorsal unpaired median neuron (DUMR7 neuron), which induces contraction of the accessory gland [5] and has a proctolin-like immunoreactivity [12]. The second type is the ordinary paired neuron (LC neuron), which has an inhibitory effect on the contraction of the accessory gland, and is likely to be serotonergic [5].

Recent investigations indicate FMRFamide immunoreactive fibers innervating the midgut of *Drosophila* larvae [8] and the dual peptidergic innervation of the blowfly hindgut by proctolin, FMRFamide and related peptides [2]. In the locust, FMRFamide and related peptides were

shown to have modulatory effects on the oviduct [6], on the heart [3], and on the foregut [1]. In the isolated locust foregut, FMRFamide was reported to potentiate relaxation induced by serotonin and to inhibit the contraction caused by proctolin [1,9].

The present report describes the presence of FMRFamide immunoreactive fibers innervating the glandular tubules of the cricket accessory gland, suggesting that an RFamide-like peptide has an important role in regulation or modulation of contractile activity of the accessory gland.

MATERIALS AND METHODS

Adult male crickets (*Gryllus bimaculatus*) reared in the laboratory, were used for the experiments. The accessory glands were fixed for 6–12 hr in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C. Rabbit anti-FMRFamide antiserum (Cambridge Research Biochemical) used for this experiment is specific to the C-terminal (RFamide), and positive reaction to this antiserum demonstrates the existence of RFamide-like peptides. Antiserum was diluted 1:2000 in phosphate buffered saline [5] (PBS, pH 7.4) with 0.5% Triton X-100 and 0.5% bovine serum albumin (BSA).

Light microscopic immunocytochemistry was employed using the avidin-biotin-peroxidase complex (ABC, Vector Lab.) method as described by Yasuyama et al [12]. Paraffin sections were incu-

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bated in primary antiserum for 48 hr at 4°C. The secondary antibody, a biotinylated goat anti-rabbit IgG, was applied to the sections at a 1:200 dilution in PBS for 1 hr at room temperature. Incubation in the PBS-diluted ABC reagent (1:100) was for 1 hr at room temperature. Each incubation was followed by three 10 min washes in PBS. The peroxidase activity was visualized by treatment with 0.05 M Tris buffer (pH=7.2) containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.005% H₂O₂. The sections were dehydrated and mounted in Canada Balsam. For whole mounts, the accessory glands were incubated in the primary antiserum for 72 hr at 4°C. Each of the solutions of the secondary antibody (1:200) and the ABC reagent (1:100) was applied for 8–10 hr at 4°C. Each incubation was followed by 12–24 hr wash in PBS. After visualization of the peroxidase activity, the preparations were dehydrated and mounted.

For electron microscopic immunocytochemistry, the post-embedding protein A-gold method was employed as described by Yasuyama et al [12]. Ultrathin sections were incubated in primary antiserum for 48 hr at 4°C. The protein A-gold complexes (Janssen Pharmaceutical; particle size 15 nm) were applied at a concentration of 1:40 in PBS with 1% BSA. The sections were stained with uranyl acetate and lead citrate, and were examined with a Hitachi H 500 electron microscope.

The specificity of the immunoreactivity was tested by preincubating the anti-RFamide antiserum with synthetic FMRFamide (Funakoshi). The test peptide was added at 10⁻⁴ M concentration to the working dilution (1:2000) of antiserum, and the solution was incubated for 24 hr at 4°C. Immunoreactivity was eliminated by such treatment.

RESULTS AND DISCUSSION

The accessory gland of the male cricket is comprised of more than 600 slender tubules arising from the lobed anterior end of the ejaculatory duct. Single-layered thin muscles wind around most of each glandular tubule's length, and multi-layered thick muscles surround the opening of each tubule to the ejaculatory duct [11]. On the

basis of the arrangement of these two types of muscles, it is likely that the antagonistic action of muscles, i.e. the contraction of single-layered muscles and relaxation of multi-layered muscles, causes the release of the secretions into the ejaculatory duct. RFamide-like immunoreactive fibers were found in both single- and multi-layered muscles (Figs. 1 and 2). In the single-layered muscles associated with a glandular tubule, the immunoreactive fibers had many varicosities and ran rather straightly along the tubule's long axis to the distal end. Projection of short thin branches from the thick fibers were observed at rather regular intervals (Fig. 1). In the multi-layered muscles surrounding the opening of each tubule, the RFa-like immunoreactive fibers ran tortuously among the muscle fibers. Figure 2 shows the immunoreactive fibers circularly arranged around the glandular tubule.

Electron microscopic immunocytochemistry revealed the widely distributed RFa-like immunoreactive axons in the accessory gland muscles. RFamide-like immunoreactivity was restricted to large electron-dense granular vesicles with a mean diameter of 141.9 nm (n=56) contained in the axons (Fig. 3). This immunoreactivity was also found in granular vesicles (141.5 nm in mean diameter, n=86) accumulated in the nerve branch (Br3) after ligation ([11]; data not shown). Our earlier investigations have revealed that Br3 carries innervation from the terminal abdominal ganglion to the accessory gland, and ligation of Br3 causes a heavy accumulation of large electron-dense granular vesicles [11, 12]. It is, therefore, suggested that RFa-like immunoreactive granular vesicles found in the accessory gland musculature are transported through the Br3.

Our recent investigation using the protein A-gold method combined with retrograde HRP labeling showed that proctolin-like immunoreactivity is present in the DUMR7 neurons innervating the accessory gland musculature [12]. The RFa-like immunoreactive fibers demonstrated in this experiment are probably derived from neurons which are distinct from the DUMR7 neurons. This conclusion is based on the following facts. First, the size of the vesicles with proctolin-like immunoreactivity found in the axons associated with the acces-

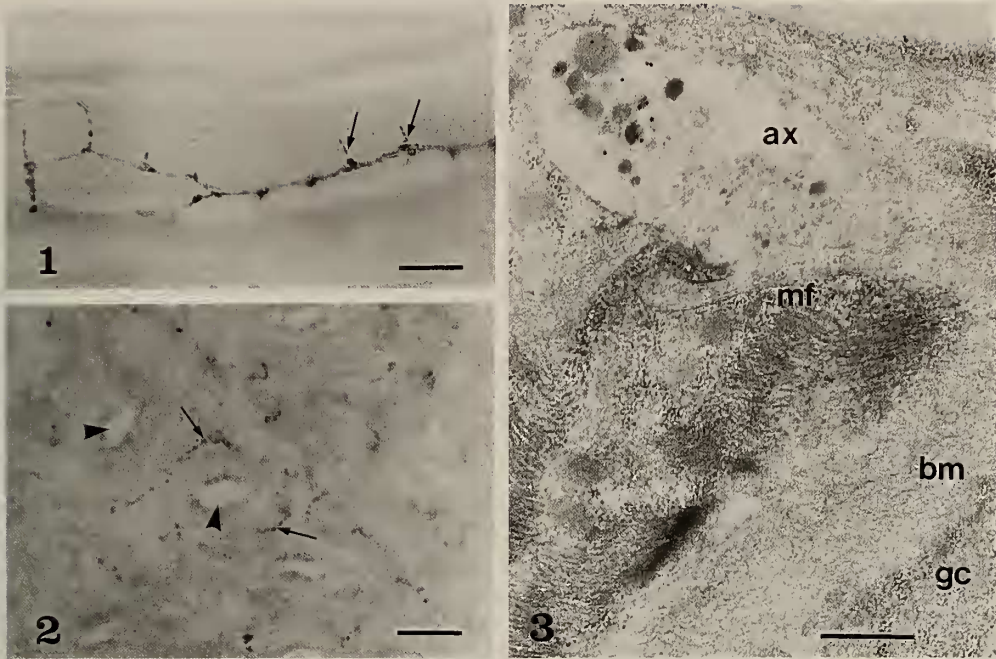


FIG. 1. RFamide-like immunoreactive fibers in a wholemount preparation of a glandular tubule of the accessory gland. Note projection of short thin branches (arrows) from the thick immunoreactive fibers. Scale bar, 20 μ m.

FIG. 2. RFamide-like immunoreactive fibers (arrows) within the proximal part of the accessory gland tubule, where multi-layered muscle fibers are located. Arrowheads indicate the lumen of the glandular tubule. Scale bar, 20 μ m.

FIG. 3. An axon (ax) containing RFamide-like immunoreactive vesicles and running within the single-layered muscle (mf) which envelops the accessory gland tubule. Immunogold labeling was restricted to the vesicles. bm, basement membrane; gc, glandular cell. Scale bar, 0.5 μ m.

sory gland musculature was 100–115 nm in diameter, while the RFamide-like immunoreactivity was restricted to the vesicles with a diameter of about 140 nm. Second, the anti-RFamide antiserum did not stain the DUMR7 neurons, but did stain the bilaterally symmetrical neurons in the terminal abdominal ganglion [10]. These suggest that a separate peptidergic innervation is supplying the accessory gland, and not only proctolin but also FMRFamide-like peptide have important roles in regulation or modulation of neuro- or myogenic activities.

In the locust, Evans and Cournil have described a pair of FLRF-like immunoreactive neurons, which are located in the suboesophageal ganglion and send their axons through the ventral nerve cord to the terminal abdominal ganglion and further, in the male locust, to the genital nerve arising

from this ganglion [4]. In the locust there is, also, a possibility that the RFa-like immunoreactivity fibers found in the accessory gland musculature derive from the neurons located in the higher ganglion. Furthermore, a possibility that the RFa-mide-like peptide is contained in the LC neurons cannot be eliminated, since anti-RFamide antiserum revealed the lateral cluster of neurons around the site where the LC neurons were located in a cluster (Yasuyama, unpublished observation). Further experiments are being carried out both to identify the RFa-like immunoreactive neurons innervating the accessory gland and to elucidate their functional roles in regulation of the mechanical activity of the accessory gland.

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